Jpn. J. Cancer Res. 87, 923–929, September 1996

Clonal Heterogeneity in Human Esophageal Squamous Cell Carcinomas on DNA Analysis

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Cancers are thought to arise through multistep accumulation of somatic mutations in the progeny of a single cell. Multiple mutations may induce molecular intratumor heterogeneity. Therefore, we examined molecular clonal heterogeneity in esophageal squamous cell carcinomas. Twenty-four esophageal squamous cell carcinomas and associated lymph node metastases were examined for microsatellite alterations, and abnormalities of the *p53* and transforming growth factor-β type II receptor (*TGF-β RII*) genes. There were eight cases (33%) showing different patterns of loss of heterozygosity in primary tumors and metastatic lymph nodes with microsatellite markers. On the other hand, the abnormalities of *p53* were identical in all these cases. No mutation was detected in the simple repeated sequences of the *TGF-β RII* gene. These results indicate that molecular clonal heterogeneity exists in esophageal squamous cell carcinomas. Therefore, care is necessary in preoperative genetic diagnosis using biopsy samples.

Key words: Clonal heterogeneity — Esophageal squamous cell carcinoma — Microsatellite alteration — *p53* — TGF-β type II receptor

With the development of molecular biological techniques, it has become widely known that cancers arise through multistep accumulation of somatic mutations in the progeny of a single cell.[1] In colorectal cancers, molecular events have been elucidated at various stages of development from adenomas to metastatic cancers.[2] Therefore, stepwise accumulation of somatic genetic changes in a tumor may induce intratumor heterogeneity.

Recently, genetic diagnosis before operation has been attempted using biopsy materials obtained on endoscopic examination. However, we should be careful about using the result of DNA analysis as an index for the choice of operation and postoperative adjuvant therapy such as radiation therapy or chemotherapy. If a tumor exhibits heterogeneity, a diagnosis involving biopsy materials may not represent the character of the whole tumor. Therefore, it is important to examine the heterogeneity of tumors. Although many studies have unraveled molecular interindividual tumor heterogeneity, few analyses have been performed to trace intraindividual clonal tumor heterogeneity.[3–7] Esophageal carcinoma is the sixth most frequent type of cancer found in males throughout the world.[8] However, no DNA analysis for tracing intraindividual clonal tumor heterogeneity in esophageal squamous cell carcinomas has been reported so far.

Microsatellite DNA is a simple repeated nucleotide sequence interspersed throughout the human genome.[9] It is very useful for the analysis of loss of heterozygosity (LOH) in various human cancers because of its high frequency of polymorphism.[10–12] The reason why we focused on the 3p region is that a high frequency of LOH in 3p has been reported in squamous cell carcinomas of the head and neck,[10,13] uterine cervix,[11,14] lung,[12,15] and esophagus,[16] suggesting the existence of a tumor suppressor gene there.

Genomic instability, also termed replication error (RER), at microsatellites is a landmark for hereditary nonpolyposis colorectal cancer and some sporadic cancers.[17–21] Although defects of a group of DNA mismatch repair genes, *hMSH2, hMLH1, hPMS1, hPMS2* and *GTBP*, are mainly responsible for this phenomenon,[22–27] the role of the genomic instability in tumorigenesis remains unclear. The incidences of RER in esophageal squamous cell carcinomas previously reported[16,28] were quite different, and thus the actual incidence and clinicopathological significance of RER in esophageal squamous cell carcinomas are uncertain. A recent report revealed that the simple repeated sequences of the TGF-β type II receptor gene (*TGF-β RII*) located on 3p21 are a primary target for genetic alteration in colon cancers that exhibit RER.[29–31] *p53* is a tumor suppressor gene, of which an abnormality is frequently detected in many cancers. The prevalence of *p53* mutations varies among tumor types, ranging from 0 to 60% in major cancers, and is over 80% in some histological subtypes. In esophageal carcinomas it is 41–50%, relatively higher than the others.[32–34] In this study, we examined genetic clonal heterogeneity between primary tumors and metastatic lymph nodes by analyzing microsatellite markers,

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p53 and *TGF-β RII*, in a series of 24 esophageal squamous cell carcinomas with lymph node metastases.

MATERIALS AND METHODS

Tissue samples Tissue samples were surgically obtained from 24 patients with esophageal squamous cell carcinomas in our department. The family histories of these patients did not indicate any hereditary diseases. None of the patients had received any therapy prior to surgery. Primary tumors, corresponding normal mucosae, and metastatic lymph nodes were frozen immediately after surgical resection and stored at −80°C until DNA extraction. Genomic DNA was prepared from frozen samples by proteinase K digestion, serial phenol and chloroform extractions, and ethanol precipitation.[35] In order to prevent contamination by normal cells, we matched the frozen samples with hematoxylin-eosin stained sections. Contamination by normal cells was less than 20% in primary tumors and almost undetectable in metastatic lymph nodes. Histologically, primary tumors and metastatic lymph nodes were the same in all cases.

Analyses of microsatellite markers Eight different dinucleotide repeat markers, D3S659,[16] D3S1029,[36] D3S1255,[16] D3S1110,[16] D2S123,[9] D2S119,[9] D10S197,[9] and D13S175,[9] were amplified using the polymerase chain reaction (PCR). In each case one primer was radiolabelled with [γ-^{32}P]ATP at the 5′-end using T4 polynucleotide kinase (Takara, Kyoto). PCR was performed in 25μl comprising 50 ng of DNA, 2.5μl of 10× reaction buffer (Biotech International, Ltd., Bentley, Australia), 0.2 m*M* of each dNTP, 0.1 units of *Taq* DNA polymerase (Biotech International, Ltd.), and 20 pmol of each primer. The reaction conditions were 94°C (1 min), 50–60°C (2 min), and 72°C (1 min) for 30 cycles. The reaction was initiated by incubation for 2 min at 94°C and ended with 10 min at 72°C. Following PCR, 3μl of the products was added to 3μl of loading buffer (95% formamide, 20 m*M* EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol), and then each sample was denatured at 85°C for 5 min and chilled on ice. Electrophoresis was performed at 1500 V for 1.5 h at room temperature on 6% polyacrylamide gels containing 7 *M* urea. After electrophoresis the gels were exposed to X-ray film at −80°C for 2–4 h, and visualized by autoradiography. Microsatellite alterations were divided into two categories, LOH and RER. Alterations were designated as LOH when the tumor DNA did not give the band corresponding to an allele which was present in normal DNA, and as RER when a band corresponding to a different molecular weight, which was not seen for normal DNA, appeared for tumor DNA. Alterations were confirmed by repeated experiments by using independent PCR products.

Analysis of *p53* alterations We used two different methods to analyze *p53*, that is, PCR-single strand conformation polymorphism (SSCP)[37] and PCR-restriction fragment length polymorphism (RFLP) analyses.[38] PCR-SSCP analysis on *p53* exons 5 to 9 was performed as described previously.[37,39] Briefly, PCR was carried out for 30 cycles. The PCR products were denatured and then electrophoresed on 10–12.5% non-denaturing polyacrylamide gels containing 10% glycerol in Tris-glycine buffer (25 m*M* Tris-HCl, 200 m*M* glycine, pH8.3). After electrophoresis, the gels were stained with silver (Daiichi Co., Ltd., Tokyo). PCR-RFLP analysis was performed on *p53* exon 4. Primers flanking a *Bst UI* RFLP within exon 4 were synthesized.[38] The PCR products were digested with *Bst UI*, and then DNA bands were detected by ethidium bromide staining following 10% polyacrylamide gel electrophoresis. LOH was defined as positive when the tumor DNA did not give the band corresponding to an allele which was present in normal DNA.

Mutation analysis of the *TGF-β RII* gene The primers used for genomic *TGF-β RII* were synthesized for nucleotide positions 677–766 and 1886–2009 containing two simple repeat sequences, $(A)_{10}$ and $(GT)_3$, respectively.[29,30] PCR-SSCP analysis was performed as described above.

RESULTS

Microsatellite alterations We examined microsatellite alterations in 24 esophageal squamous cell carcinomas accompanied by lymph node metastases. We used eight microsatellite markers, D3S659, D3S1029, D3S1255, D3S1110, D2S119, D2S123, D10S197 and D13S175. Representative results as to LOH and RER are shown in Fig. 1. The frequencies of LOH and RER at each locus are summarized in Table I. There was no case showing both LOH and RER.

LOH was observed in 8 (33%) of the 24 esophageal squamous cell carcinomas using eight microsatellite markers, some of which showed LOH in more than one marker (Table I). LOH was detected in 30% (7 of 23) at the four loci on 3p. In particular, the frequency of LOH at D3S659 (3p13) was 50%, i.e., higher than that at the other loci. LOH was only observed in primary tumors, i.e., not in metastatic lymph nodes, in seven of the eight cases showing LOH (Fig. 1A and Table II). Another case (9247) only showed LOH in the metastatic lymph node. Therefore, all of the eight cases have intraindividual heterogeneity. There was no statistically significant correlation between LOH at each locus and clinicopathological findings, such as age, sex, histology, location, tumor size, depth of invasion, and prognosis.

RER was observed in 12.5% (3 of 24) of the esophageal squamous cell carcinomas (Fig. 1B and Table I).

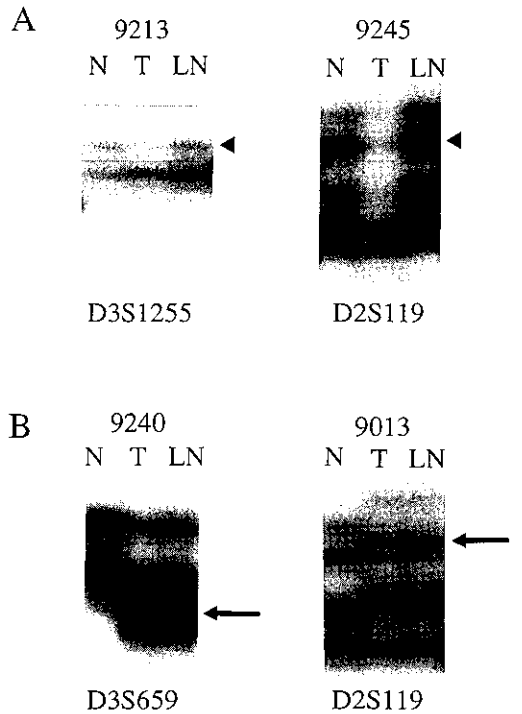

Fig. 1. Representative results on microsatellite alterations. A, Cases with LOH. Lost alleles are indicated by arrowheads. B, Cases with RER. The mobility shifts indicated by arrows are seen in both T and LN. The numbers at the top are patient numbers. The numbers at the bottom are microsatellite markers. N, normal tissue; T, primary tumor; LN, metastatic lymph node.

Table I. Microsatellite Alterations in Esophageal Squamous Cell Carcinomas

Marker	Chromosomal location	Incidences of alteration (%)	
		LOH	RER
D3S 659	3p13	50 (7/14[a])	4.2 (1/24[b])
D3S1029	3p21.2-21.31	8.3 (1/12)	0 (0/23)
D3S1255	3p24.2-25	25 (2/8)	0 (0/24)
D3S1110	3p25.1-25.3	30 (3/10)	0 (0/23)
Subtotal (3p)		30 (7/23)	4.2 (1/24)
D2S123	2p16	0 (0/15)	0 (0/18)
D2S119	2p16-21	8.3 (1/12)	9.1 (2/22)
D10S197	10p	25 (1/4)	0 (0/21)
D13S175	13p	33 (1/3)	0 (0/20)
Total		33 (8/24)	12.5 (3/24)

a) Positive cases/informative cases.
b) Positive cases/determined cases.

In all cases, RER was only recognized in one marker, that is, there was no case showing RER in more than one marker. The electrophoretic patterns were the same in primary tumors and metastatic lymph nodes in all microsatellite alteration-positive cases (Fig. 1B). There was no significant correlation between RER and clinicopathological findings.

Table II. Heterogeneity in Esophageal Squamous Cell Carcinomas

Case	Microsatellite LOH			*p53* SSCP		*p53* LOH	
	Markers[a]	T[b]	LN[b]	T	LN	T	LN
9011	A, B	+	−	−	−	+	+
9014	A, D	+	−	−	−	−	−
9207	A, C	+	−	−	−	−	−
9213	A, C, D	+	−	+	+	+	+
9227	A, F	+	−	−	−	−	−
9230	A	+	−	+	+	−	−
9245	E, G	+	−	+	+	NI[c]	
9247	A	−	+	−	−	NI	

a) A: D3S659, B: D3S1029, C: D3S1255, D: D3S1110, E: D2S119, F: D10S197, G: D13S175.
b) T: Primary tumor, LN: metastatic lymph node.
c) NI: Not informative.

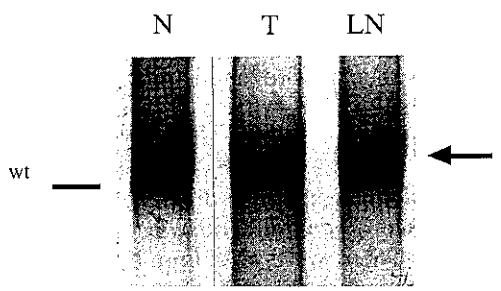

Fig. 2. PCR-SSCP analysis of exon 8 of the *p53* gene in patient 9230. The PCR products were electrophoresed on a 12.5% non-denaturing polyacrylamide gel containing 10% glycerol and then stained with silver. The mobility shifts indicated by an arrow are seen in both T and LN. N, normal tissue; T, primary tumor; LN, metastatic lymph node.

***p53* Alterations** We searched for abnormalities of *p53* to elucidate the heterogeneity between primary tumors and metastatic lymph nodes in these 24 samples. We examined exons 5 to 9 of *p53* on PCR-SSCP analysis. These areas contain hot spots for mutations in many carcinomas.[40] Mutations of *p53* were found in 42% (10 of 24 cases) of the esophageal squamous cell carcinomas as shown in Fig. 2. As for the details, there were six cases showing mutations in exon 5 and four in exon 8. LOH at exon 4 of *p53* was found in 24% (3 of the 14 informative cases) of the esophageal squamous cell carcinomas on PCR-RFLP analysis. A representative result as to the LOH of *p53* is shown in Fig. 3. The patterns of these *p53* abnormalities in primary tumors and metastatic lymph

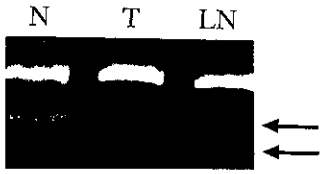

Fig. 3. PCR-LOH analysis of the *p53* gene. *Bst UI* digestion of the PCR products of *p53* exon 4 from patient 9011 were electrophoresed on a 10% polyacrylamide gel and then stained with ethidium bromide. RFLP can be seen in case 9011, and the LOH indicated by arrows can be recognized in both T and LN. N, normal tissue; T, primary tumor; LN, metastatic lymph node.

nodes were identical in all cases with abnormalities (Table II). There was no significant correlation between *p53* abnormalities and clinicopathological findings.

***TGF-β RII* mutations** When we examined the *TGF-β RII* gene by PCR-SSCP analysis, no abnormality of *TGF-β RII* was found at the two simple repeated sequences in all 24 cases (data not shown).

Clonal heterogeneity From the combination of the results shown above, there were four cases which showed the same *p53* abnormalities and different LOH patterns in one or more microsatellite markers between tumors and lymph nodes. There were also four cases showing the different LOH results using microsatellite markers between tumors and lymph nodes, but no *p53* abnormality (Table II).

DISCUSSION

The frequencies of RER in esophageal squamous cell carcinomas previously reported were quite different. It has been shown that the frequency of RER on 3p was 60% (21 of 35 cases).[16] On the other hand, Meltzer *et al.* found that it was only 2% (1 of 42 cases).[28] Others reported that the frequency of RER was 6.1% (2 of 33 cases) in tumors of patients with multiple primary cancers of the esophagus and various other tissues.[41] We used eight microsatellite markers in our study, three of which were the same as those used in the previous study, i.e., CI3-1169(D3S1110), D3S1255 and CI3-373(D3S659), and which showed frequent alterations.[16] However, in our study the incidence of RER in esophageal squamous cell carcinomas was 12.5% (3 of 24 cases), and all of them only had RER in one marker. The criteria for RER (+) are generally thought to be that samples should be examined at more than four microsatellite markers and should show abnormalities at more than one marker. Judging with these criteria, there was no case showing RER(+) in our study. When we examined RER in 51 esophageal squamous cell carcinomas including these 24 cases, five cases (9.8%) only showed RER in one marker (unpublished observation). Therefore, we conclude that the incidence of RER in esophageal squamous cell carcinomas is low. The difference between the previous study[16] and ours might have arisen because these three markers (D3S1110, D3S1255 and D3S659) often gave unclear abnormal bands, and many of them did not give reproducible results. We judged that cases without reproducibility were negative.

TGF-β is a multifunctional growth factor that regulates cell differentiation and the expression of extracellular matrix proteins, and also inhibits the growth of many cells, including those of epithelial cell type.[42] This growth inhibitory signal is transduced by two transmembrane serine/threonine kinases called receptors I and II.[43] Recently, it was reported that frequent inactivation of the *TGF-β RII* gene occurred in RER (+) sporadic and hereditary nonpolyposis colorectal cancer cell lines, which suggested that *TGF-β RII* may be a major target gene of defective DNA repair.[29–31] When we examined *TGF-β RII* in two regions where mutations have been reported, there was no mutation in *TGF-β RII* in any case, including the three cases showing RER in one microsatellite marker. When we also analyzed *TGF-β RII* mutation in 27 more esophageal squamous cell carcinomas, there was no case showing mutation. The results of RER and *TGF-β RII* suggest that defects of DNA mismatch repair genes have little, if any, influence on esophageal carcinogenesis. The incidences of LOH at the examined loci on 3p were: D3S659, 50%; D3S1029, 8.3%; D3S1255, 25%; and D3S1110, 30%. This result is similar to that of a previous study on esophageal carcinomas.[16] LOH in 3p was also high (79%) in squamous cell carcinomas of the head and neck.[10] These data suggest the existence of gene(s) in 3p involved in esophageal carcinogenesis. Further studies are necessary to clarify the involvement of the 3p region in esophageal carcinogenesis.

As for the heterogeneity of esophageal cancers, we obtained interesting LOH results in this study. Seven of the eight cases only showed LOH in primary tumors, i.e., not in metastatic lymph nodes. The other case was the opposite, that is, LOH was only recognized in metastatic lymph nodes. These data indicate that there is heterogeneity between primary tumors and metastatic lymph nodes in esophageal squamous cell carcinomas. Since LOH was not observed using microsatellite markers in the metastatic lymph nodes of seven cases, it is necessary to confirm the presence of cancer cells in metastatic lymph nodes. We then analyzed *p53* mutations in the 24 cases. There were 10 cases (42%) with *p53* mutations, which were identical in primary tumors and metastatic lymph nodes in all 10 cases. LOH of *p53* was observed in three of the informative 14 cases, and was again observed

in both primary tumors and metastatic lymph nodes. Therefore, four of the eight cases with heterogeneity of microsatellite LOH exhibited the same clonality in the primary cancers and metastatic lymph nodes as regards abnormalities of *p53* (Table II), indicating that cancer cells were present in these metastatic lymph nodes. Our results suggest that esophageal squamous cell carcinomas acquire heterogeneity in the process of progression, like other cancers.[3–7]

Mutations acquired at an early stage of carcinogenesis may be shared by most tumor cells, while mutational hits acquired at later stages of clonal tumor evolution would be restricted to particular tumor subclones or morphologically distinct tumor areas.[5] According to this hypothesis, abnormalities of *p53* may be an early change, whereas LOH detected by microsatellite markers might occur at a later stage in esophageal carcinogenesis. Interestingly, LOH was observed using microsatellite markers in primary esophageal carcinomas, but not in metastatic lymph nodes, which represent a more advanced stage of carcinogenesis. This phenomenon was also observed for *p53* mutations and RER in some gastric cancers,[6] and in an LOH study on prostate cancers.[5] It remains unknown why this phenomenon occurred. One interpretation of this phenomenon is that an abnormality occurring at an early stage of carcinogenesis becomes unnecessary and thus disappears at a later stage. Alternatively, an allelic loss event might occur in the primary tumors after metastasis, or the metastasis might arise from another unsampled tumor focus.

The heterogeneity of esophageal squamous cell carcinomas was demonstrated in this study. The presence of intraindividual heterogeneity means that, if esophageal squamous cell carcinomas acquire heterogeneity with progression, it will be uncertain whether a genetic diagnosis based on a part of the tumor tissue represents the nature of the whole cancer or not. This must be borne in mind in connection with preoperative genetic diagnosis using biopsy samples.

ACKNOWLEDGMENTS

We wish to thank Dr. Nagai for advice regarding the pathological study. This work was supported in part by a Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan.

(Received May 1, 1996/Accepted June 17, 1996)

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